Effects of Acute Cadmium Exposure on the Pituitary Gland of *Podarcis sicula*

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Abstract: Reptiles are rarely used in studies on the possible toxic effects of heavy metals even if they are susceptible to the accumulation of persistent pollution due also to their presence in a variety of habitats. Cadmium is a heavy metal, a significant environmental pollutant and an endocrine disruptor. Therefore the aim of this study was to analyze the cytotoxic effects of cadmium on the pituitary gland of the lizard *Podarcis sicula* after an acute exposure to this metal. The analysis were carried out after 2, 7 and 16 days following the intraperitoneal injection of a single and massive dose of cadmium chloride. The pituitary glands were analyzed by histological and immuhistochemical stains. Besides cadmium accumulation in brain was measured by atomic absorption spectrometry. Cadmium concentration increased in lizard brain lightly after 2 days and widely after 16 days. The tissue of the pituitary gland appeared slightly atrophied in a few areas only at 7 and 16 days after treatment. Moreover an increase in intensity of immunostaining and occurrence of some adenohypophyseal cells was revealed respect to control lizards. This evidence suggests an inhibitory effect of cadmium on the normal hormonal secretion. Evidently an acute cadmium exposure in *P. sicula* involves the accumulation of this metal in the brain but also the alteration of the normal endocrine function of the pituitary gland.

Keywords: Cadmium, pituitary gland, lizard, adenohypophyseal cells.

INTRODUCTION

Non-biodegradable metals persist in the environment for a long period and cause serious ecotoxicological problems [1]. Among the heavy metals cadmium (Cd) is considered one of the most toxic trace elements in the environment [2]. Its many industrial uses result in large dispersion throughout ecosystems [3]. Cd enters into the food chain and it is accumulated over time in blood, kidney, and liver [4-6] as well as in the reproductive organs and also in the hypothalamus and in the pituitary gland of mammals [7-10]. Cd has also been shown to affect cell physiology and growth [11, 12] and to induce lipid peroxidation (LPO), that is dependent on oxygen free radicals [13, 14]. In addition to its well-known toxic effects, cadmium is now also regarded as potential endocrine disruptor [15, 16]. Endocrine disrupting chemicals (EDCs) are natural or synthetic agents that can mimic, enhance or inhibit the action of the endogenous hormones [17]. Exposure to this metal is associated with changes in the activity of endocrine system in male and female rats [18]. It's reported that Cd, at very low concentrations, influences the levels and circadian variations of pituitary hormones in plasma of rat [19, 12]. Moreover cadmium exposure differentially affects circulating concentrations of prolactin, adrenocorticotropic and growth hormones [20]. The morning levels of prolactin decreased after exposure to cadmium in rats, independently of the dose of cadmium used or route of administration [12, 20]. Different studies have also shown that cadmium affects plasma gonadotropin levels [21, 22]. Experiments executed on the monkey *Presbytis entellus entellus* have evidenced that cadmium provokes consequently changes in FSH and LH cells of the adenohypophysis, inducing testicular necrosis [23]. In the catfish *Clarias batrachus*, the exposure to cadmium chloride caused a significant increase in the ACTH cell nuclear indices at day 7, 14 and 28, whereas the thyrotropin and gonadotropin secreting cells showed inactivation and accumulation of secretory products [24]. In the mouse *Mus Platythrix* cadmium induces hypertrophy and hyperplasia of pituitary gonadotrophs [25]. Poliandri *et al*. showed that Cd induces apoptosis in pituitary cells and directly inhibits PRL release [26]. Given that cadmium is high concentrate in the soil, the reptiles result exposed to this metal probably by eating contaminated preys. Therefore reptiles could serve as useful bioindicators because many species are wide-spread, relatively long-lived, occur in a variety of habitats and inhabit a range of trophic levels [27, 28]. Yet, reptiles are rarely included in studies of environmental contamination and ecological risk assessments. While there are some data on contaminant levels in reptiles [28-30], very few experiments were conducted on hormone secretion in reptiles. Lizards, as other reptiles, are ectothermic, with low metabolic rate. Additionally, they have relatively simple enzyme systems and a poor ability to detoxify pesticides absorbed, inhaled or ingested [31]. Therefore, the common lizard *Podarcis sicula* seems to be a good model as laboratory reptile species for toxicological investigations. Previously in lizard we reported that a chronic and oral exposition at low concentrations of $CdCl_2$ for 120 days affects, by an inhibitory effect, FSH and

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LH hormonal secretion which returns at normal values at 120 days and by evident changes of the general morphology of the gland for the progressive disorganization of the endocrine tissue [32]; moreover a single high intraperitoneal dose of $CdCl_2$ induces apoptosis, in particular in the rostral pars distalis, and this effect appears irreversible [33]. Therefore the present study was undertaken to assess whether acute exposure to cadmium through a single intraperitoneal injection may also alter the adenohypophyseal cells and the morphology in the pituitary gland of adult *Podarcis sicula* lizards.

MATERIALS AND METHODS

Thirty-six adults of *Podarcis sicula* lizards were captured in captivity near Naples (Italy) and kept under controlled conditions of light and temperature. Food and water were available ad libitum. One group was treated by an intraperitoneal injection of a single and massive dose (2,0 mg/kg body weight) of cadmium chloride ($CdCl_2$); the other group received an injection with a physiological saline solution to use as control. After 2, 7 and 16 days animals were killed under anaesthesia by decapitation. Experiments were performed with the approval of institutional committees: all efforts were made to avoid animal suffering and to minimize the number of animals used.

1. Atomic Absorption Spectrometry (AAS)

Sixteen specimens were used for the determination of total cadmium content in *P. sicula* brains from control and Cd-treated lizards. The brains were weighted and quickly frozen at -80°C. The method used was reported in 'APAT IRSA-CNR Sez. 3000'-"Metals and metal species"-division 3010 "Preliminary treatment of samples for heavy-metal analysis through acid mineralization".

The samples were digested with a hot concentrated nitric acid in an open flask under the hood for the solubilization of metals. The heavy metals obtained in this way were then analyzed by atomic absorption spectrometry in flame using a Varian AA280FS Atomic Absorption Spectrometer.

2. Tissue Preparation

The gland of twenty lizard, five in each group, was studied in toto with the brain: after removal of the skullcap, the brains were fixed in Bouin's solution for 48 hours at room temperature and then decalcified in a solution of 5% EDTA in 10% formalin for 25-30 days, dehydrated and enclosed in paraffin. Serial sagittal sections of 6 µm were processed for routine histological and immunohistochemical staining. Mallory's trichromic stain was used for the study of the general morphology while the immunohistochemical procedure was applied for the identification and the observation of the adenohypophyseal cells.

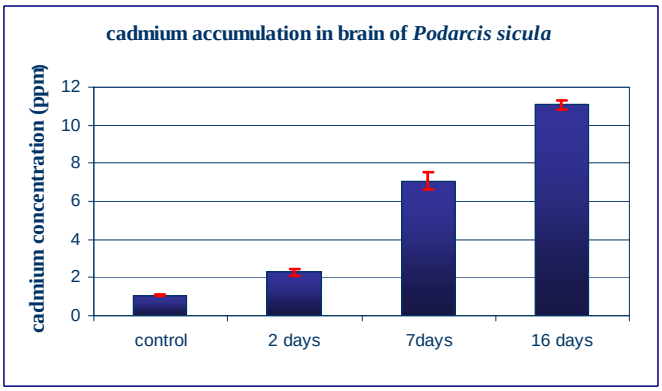
3. Immunohistochemistry

For immunohistochemical staining [34], sections were processed according to the ABC technique [35] using the following heterologus antisera at specific working solution: anti-human LH (1:100, Signet Laboratories, MA), anti-human FSH (1:100, Signet Laboratories, MA), anti-human PRL (1:300, Signet Laboratories, MA), anti-syntethic $ACTH^{1-24}$ (1:600, Biogenesis, UK). Visualization was carried out using the Vectastain Elite ABC kit (Vector, USA) and revealed by 3 mg 3,3'-diaminobenzidine-tetrahydrochloride (Sigma, USA) in 10 ml PBS and 150 µl 3% H_2O_2. These slides were stained with hemalum for 1 minute. Antibody specificity was assessed by omitting the primary antisera and by absorbing each antiserum with the specific hormone. The images were examined and acquired by a Kontron Electronic Imaging System KS300 (Zeiss, Germany). The quantifica-tion of the different cellular types was carried out on at least 300 cells on serial sections per animal and relative to specific regions as the rostral, medial and caudal pars distalis. Data were expressed as number of immunostained cells x 100/number of total cells.

RESULTS

The determination of the cadmium concentration in the brains by atomic absorption spectrometry showed an accumulate of this metal for the treated animals. In the time the concentration of cadmium in the brain of treated lizards appeared to increase exponentially and it was maximum after 16 days (2 days: 2.27 ± 0.17 ppm; 7 days: 7.08 ± 0.45 ppm; 16 days: 11.06 ± 0.22 ppm), as reported in the graphic of Table **1**. However traces of cadmium (1.07 ± 0.02 ppm) was also revealed in control lizard brains. No effects were observed on body weight gain and the animals appeared to be in good physical condition at the end of the experiment.

Table 1. Cadmium Concentrations in Brain of *P. sicula* to Time Zero (Control) and After 2, 7 and 16 Days of Cadmium Exposure



Cadmium concentrations, determined by atomic absorption spectrometry, as described under methods, is reported as the mean ± S.D.

Histology

P. sicula pituitary gland appeared extended in the cephalic-caudal direction in which the pars distalis (PD) was organized in a rostral pars distalis (RPD), a caudal pars distalis (CPD) and a medial pars distalis (MPD) with homogenous and greatly vascularized cellular cordons. In control specimens the pituitary gland appeared compact and with the adenohypophyseal cells clearly identifiable by trichromic stain. In lizards treated for 2 days, the morphology of the pituitary gland was similar to that of the control specimens. Indeed the tissue of the pituitary gland appeared slightly atrophied in a few areas in the lizards at 7 days as well as at 16 days after treatment (Figs. **1a**, **1b**).

Immunohistochemistry

ACTH Cells

ACTH cells were observed in the all RPD (29 ± 0.07%), as well as in the PI (59 ± 0.12%) and also in MPD (12 ±

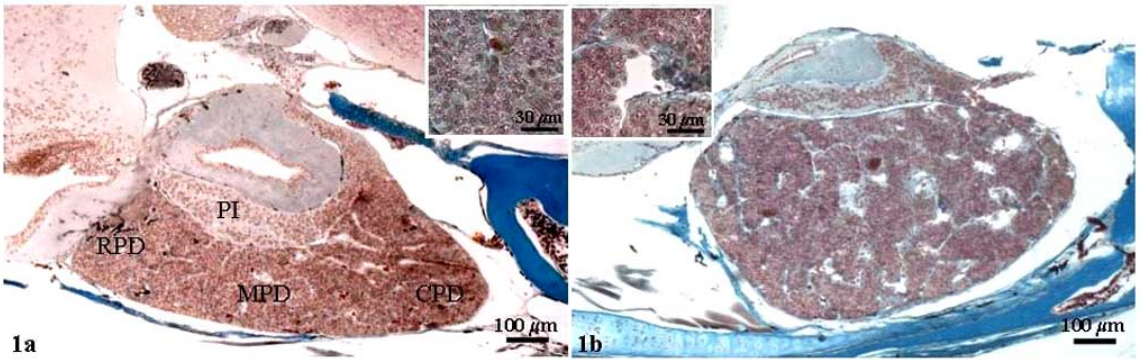


Fig. (1). Mallory stain. Sagittal sections of *P. sicula* pituitary gland. **1a**: Control lizard showing the extension of the gland in the cephalic-caudal direction and the subdivision of the pars distalis in a rostral pars distalis (RPD), a medial pars distalis (MPD) and a caudal pars distalis (CPD). PI is the pars intermedia. The PD appear with compact cordons and with the adenohypophyseal cells clearly identifiable (see detail). **1b**: Treated lizard at 16 days, in which the gland appears atrophied in different areas of the PD of which in the detail is reported a particular.

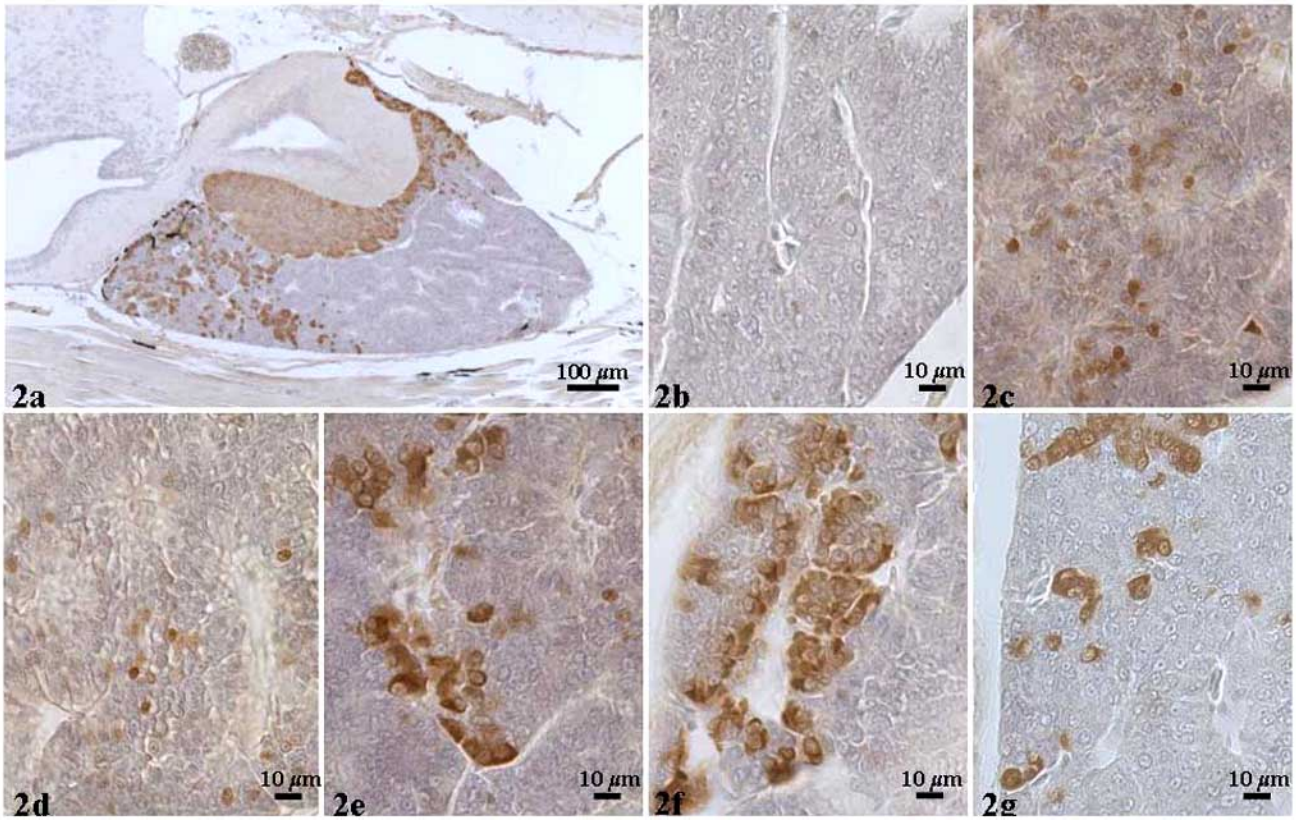


Fig. (2). ABC technique, ACTH cells (in brown). **2a**: Control lizard, showing the occurrence of ACTH cells in the RPD, MPD and PI. Note the absence of these cells in CPD of which in Fig. (**2b**) is reported a detail. **c**: CPD of treated lizard at 2 days to note the copious occurrence of these cells. **2d**: CPD of treated lizard at 16 days. **2e**: MPD of treated lizard at 2 days. **2f**: Treated lizard at 16 days to observe in MPD the increase of ACTH cells but with a morphology similar to them of control specimens in Fig. (**2g**).

0.13%) (Fig. **2a**). They were elongated in shape, with a central nucleus and a marked cytoplasm. These cells appeared indeed absent in CPD of the control specimens (Fig. **2b**). After 2 days of treatment these cells appeared meaningfully also in the CPD (16 ± 0.1%) with a strong immunostain of their cytoplasm (Fig. **2c**); they were revealed also at 16 days (13 ± 0.23%) but isolated and little in the shape without a cordonal organization (Fig. **2d**). The increase of ACTH cells was revealed also in the MPD at 2 (22.5 ± 0.04%) (Fig. **2e**) and 16 days (24 ± 0.05%) (Fig. **2f**) but with a similar morphology to them of controls (Fig. **2g**).

PRL Cells

In control lizards these cells appear pyriform or ovoidal in shape, with an eccentric nucleus and a moderately dense cytoplasm. They were isolated or organized in cellular cordons of small dimensions and occurrence in the RPD (24 ± 0.05%), and MPD (22 ± 0.24%) (Fig. **3a**). PRL cells

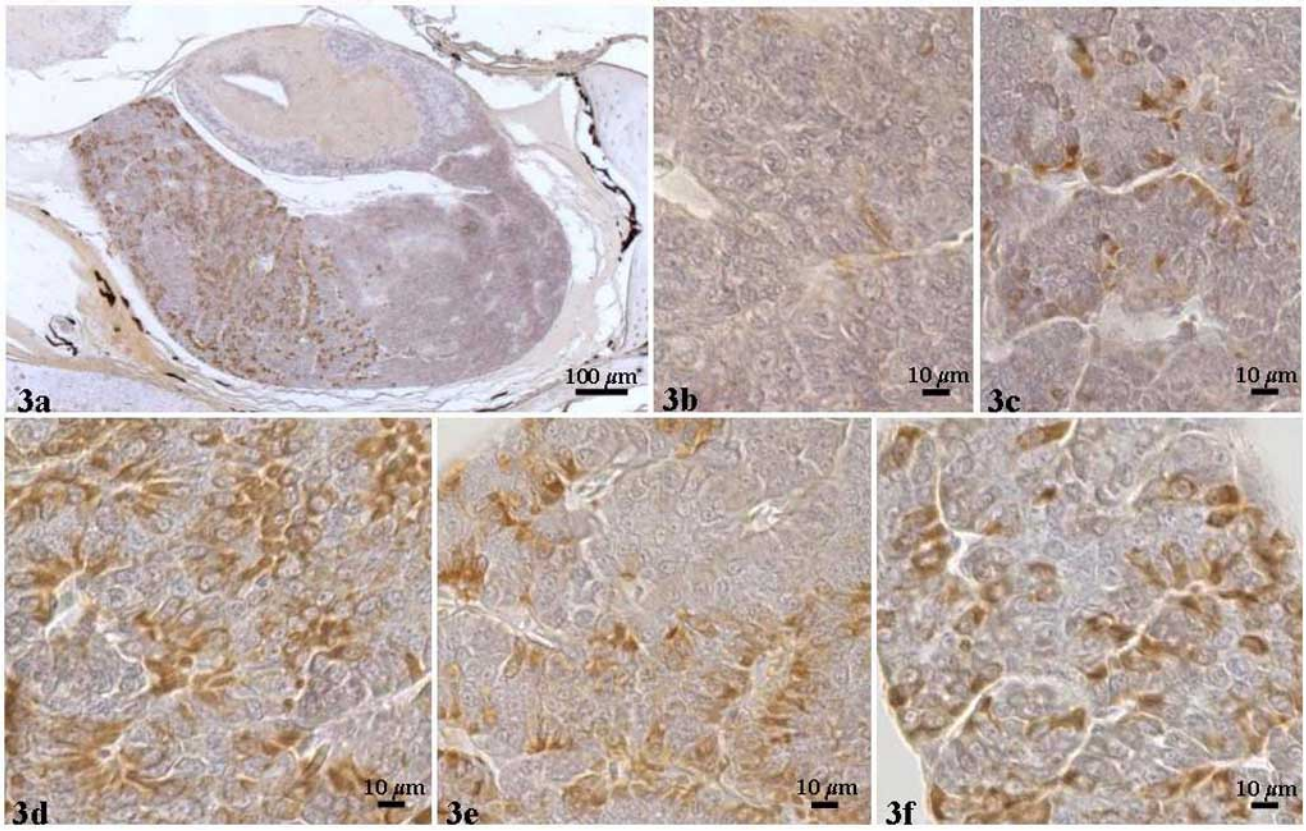


Fig. (3). ABC technique, PRL cells (in brown). **3a**: Control lizard, showing the occurrence of PRL cells in the RPD and in the MPD. **3b**: Control lizard, showing the absence of PRL cells in CPD. **3c**: In treated lizard at 2 days these cells appear also in CPD, but more copious after 7 days in Fig. (**3f**). **3d**: Treated lizard at 7 days, to compare the increase of PRL cells in MPD with those of the control in Fig. (**3e**).

appeared rare in the CPD (1.2 ± 0.07%) (Fig. **3b**) and absent in the PI. A little increase appears at 2 days after the injection in the CPD (13 ± 0.05%) (Fig. **3c**). In the treated lizards these cells appeared more numerous at 7 days in the MPD with an increase in order to 0.6 ± 0.07% respect to the control (Figs. **3d**; **3e**) but extending more copious also in the CPD (29.7 ± 0.07%) (Fig. **3f**). No particular morphological changes were revealed.

FSH Cells

FSH cells were generally and principally localized in the CPD (45 ± 0.05%) (Fig. **4a**); they appeared very small, round and organized in cellular cordons of medium dimensions (Fig. **4b**). A lot of them were also observed in the MPD (32 ± 0.07%), but rarely in the RPD (5.3 ± 0.09%) (Fig. **4c**). These cells increase particularly in RPD, after 2 days in order of 14 ± 0.1% (Fig. **4d**) and after 16 days in order to 18 ± 0.1% (Fig. **4e**). In the CPD, at 16 days, they appeared also more large in the shape with a moderately dense cytoplasm (Fig. **4f**).

LH Cells

LH cells were smaller, in comparison with FSH positive cells, and less intensely immunostained. The LH cells were round or oval in shape with a central nucleus. These cells were little, isolated and distributed mainly in the CPD (22 ± 0.12%) (Fig. **5a**), extending in the MPD (Fig. **5b**) (18 ± 0.06%). Little presence of LH cells was observed in RPD (12 ± 0.11%) (Fig. **5c**). Anti-LH positive cells were isolated, without a cordonal organization or grouped in very small islets in the periphery of glandular cords. After 2 days an increase of cellular number was observed only in RPD (22.2 ± 0.3%) (Fig. **5d**). At 7 days the distribution of LH cells showed a strong increment in RPD (32.5 ± 0.1%) (Fig. **5e**) and also in the MPD (28.7 ± 0.1%) (Fig. **5f**).

DISCUSSION

In *P. sicula* an intraperitoneal injection of a single and massive dose of cadmium chloride changes moderately the general morphology of the gland: the tissue appears slightly atrophied in a few areas in the lizards at 7 but above all at 16 days, when the concentration of this metal in the brain is maximum. These morphological alterations aren't so expressive like the observations that we have just reported regard the changes of the pituitary gland in lizard after an oral and chronic exposure to $CdCl_2$ at a lower concentration. In fact in lizards, exposed to an oral and chronic treatment, the pituitary tissue appeared meaningfully atrophied in some areas, with wide and irregular intercellular spaces and with several cells altered in the shape and more expressed at 90 and 120 days [32]. This evidence has been reported also in other glandular tissues as in the thyroid of the catfish *Clarias batrachus* [24], in the testis of the cyprinid *Puntius sarana* [36] and of the monkey *Presbytis entellus entellus* [23]. Therefore, morphological alterations induced by cadmium on the lizard are different if the exposition is relative to a single but acute intraperitonel treatment or to a chronic oral treatment with a lower dose.

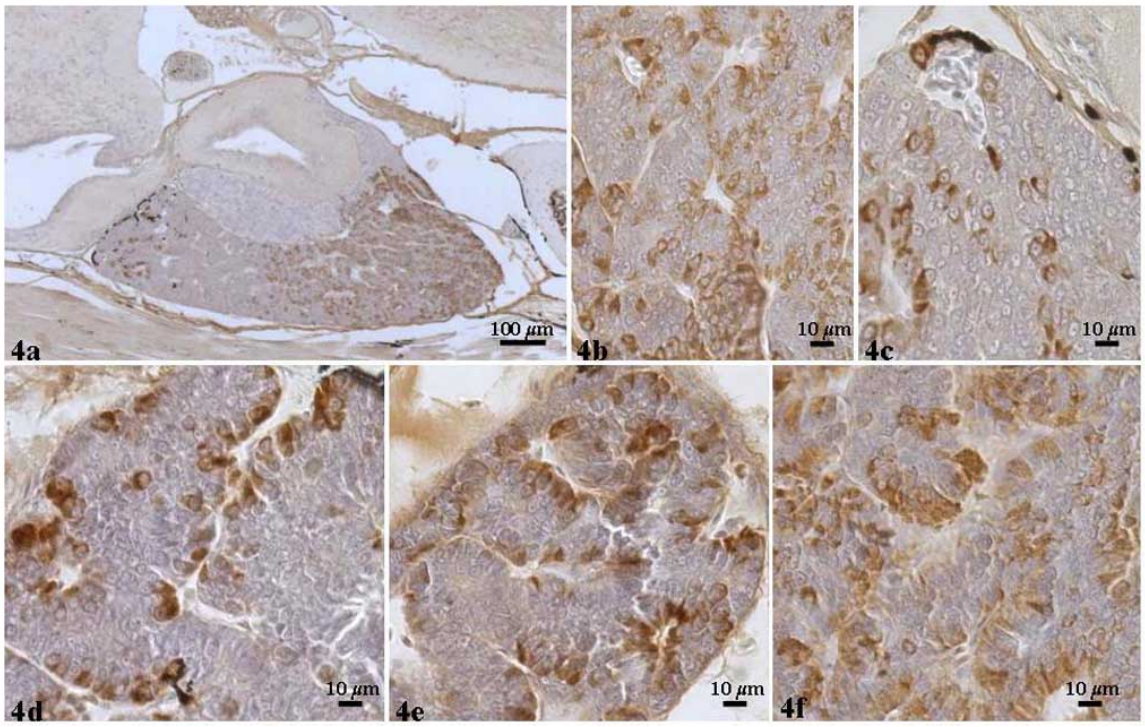


Fig. (4). ABC technique, FSH cells (in brown). **4a**: Control lizard, showing the occurrence of FSH cells principally in the CPD, but also in the MPD and in the RPD. **4b**: Note the shape and the distribution of these cells in CPD. **4c**: In control lizard FSH cells appear few expressed in the RPD. **4d**: RPD of treated lizard at 2 days. **4e**: RPD of treated lizard at 16 days, to note the considerable increase of FSH cells in this area. **4f**: In CPD of treated lizard the cells appear more large in the shape with a moderately dense cytoplasm respect to them of the control specimens reported in Fig. (**4b**).

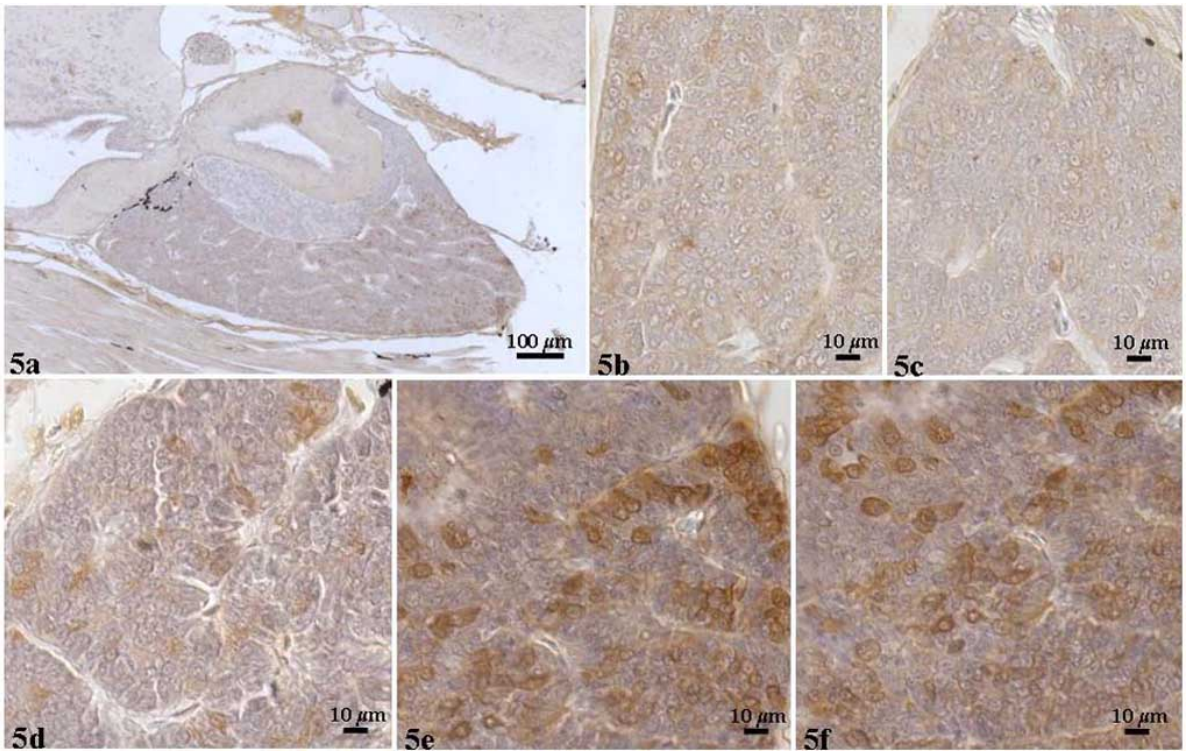


Fig. (5). ABC technique, LH cells (in brown). **5a**: Control lizard, showing the LH cells distributed mainly in the CPD, extending in the MPD of which a detail in Fig. (**5b**). **5c**: Scarce LH cells are in RPD of control lizard. **5d**: Treated lizard at 2 days in which is evident an increase of cellular number in RPD. **5e**: At 7 days in RPD the distribution of LH cells shows a strong increment. **5f**: This increase is appreciable also in the MPD.

However also in this study we have observed that cadmium have a cytotoxic effect on the gland with an evident alteration of cellular expression of adenohypophyseal cells by an inhibition of secretion of ACTH, PRL, GTH cells. The increase in number of these cells, their occurrence in regions where generally they are absent or few expressed and the increase of immunostain of their cytoplasm are indicative of an inhibitory effect of cadmium on all these cells. We have also reported as in the lizard a single high intraperitoneal dose of $CdCl_2$ induces apoptosis, like also in the anterior pituitary cells of the rat [37], and this effect appears irreversible [33]. It's evident that also in lizard cadmium has an important destructive action on the physiology of adenohypophyseal cells. A single and acute exposition to cadmium is more toxic respect a chronic treatment for 120 days in which the GTH cells, whose secretion is initially inhibited, return indeed at normal values [32]; datum that could be displayed like a probably adaptation to toxic action of cadmium in the time, when the dose of cadmium isn't very high. Indeed a single high intraperitoneal dose of $CdCl_2$ induces dysregulation of ACTH, PRL and GTH cells in lizard pituitary gland. In fact these cells increase in number but appear also in regions of gland in which generally they are absent; this cellular alteration is associated also with irreversible apoptotic processes just reported [33].

In any case the inhibitory effect of cadmium on the hormonal secretion of many adenohypophyseal cells has been just reported in mammals: the levels of LH, PRL and GH in serum of the rat exposed to cadmium decrease [20, 22] as well as the levels of GTH in pig [38] and female rats [39]. Recently it has been also reported that Cd modifies the lactotrophs activity of pituitary gland through biochemical, genomic and morphological changes, contributing directly or indirectly to the levels of serum prolactin in rat [53]. In mammals Cd differentially affects the secretory mechanisms of the pituitary hormones: the effects of this metal are dose-dependent only for prolactin and ACTH [12]. In the fish *Puntius sarana* [36] is reported that only high concentrations of $CdCl_2$ influence the pituitary gonadotrops with a gradual accumulation of secretory granules. Pituitary secretion activity has been shown to be affected by metals [40] and this endocrine gland is a particularly sensitive target to cadmium toxicity [19, 26, 41], but few it's known regard the mechanism of action of the Cd like endocrine disruptor. It's just reported that divalent cations, as Cd, inhibit *in vitro* release of GH and PRL from bovine adenohypophyseal secretory granules [42]. Calderoni *et al.* [43] report that cadmium modifies the lipid contents of pituitary gland and directly or indirectly the levels of prolactin and growth hormone in serum. The importance of the effect of Cd on lipids lies in the fact that they are components of cell membranes that act as a barrier between the cell and its environment. On the other hand, it is known that phosphatidic acid and phospholipase D are involved in the regulation of the hormone secretion in endocrine cells [44] and that phosphatidic acid obtained from phosphatidy-lcholine is necessary for the secretion in endocrine cells [45]. The inhibition of phosphatidic acid synthesis alters the structure of Golgi apparatus and inhibits endocrine cell secretion [46]. Lafuente *et al*. [47] have reported that the inhibitory effect of cadmium on PRL and LH secretion may be partially explained by a decrease in the content of glutamate and aspartate in anterior hypothalamus. Several studies have shown that cadmium could compete with calcium at the pituitary level [48, 49], which results in altered calcium regulation. Thus it either interferes with calcium influx through the membrane channel [50-52] and alters intracellular calcium mobilization. Further researches are certainly required to define the mechanism of cytotoxic action of cadmium on the pituitary cells. In lizard we have showed a direct correlation between the accumulation of this metal in the brain and the alteration of the normal occurrence and distribution of the ACTH, PRL and GTH cells and their secretory activity as just reported in literature in mammals [10, 20]. Moreover *P. sicula* shows to be a good model for future studies to define the action of this metal on the pituitary gland.

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